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Removal of Antibiotic Resistance Genes in Two Tertiary Level Municipal Wastewater Treatment Plants

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Abstract

Raw wastewater can contain high levels of antibiotic resistance genes (ARGs), making municipal wastewater treatment plants (WWTPs) critical for the control of the release of ARGs into the environment. The objective of this study was to investigate how individual treatment steps in two tertiary WWTPs affected the removal (copies/mL) and relative abundance of ARGs (copies/copies 16S rRNA genes). Nine ARG markers, representing resistance to commonly used antibiotics, as well as one integron gene (intI1) to assess ARG mobility potential, were quantified using quantitative real-time PCR (qPCR). Both WWTPs met provincial effluent regulations for removal of carbonaceous oxygen demand (CBOD₅) and total suspended solids. Eight of the ten ARG markers (intI1, sul1, sul2, tet(O), ermB, blaCTX-M, blaTEM, qnrS) were detected in all samples. In contrast, mecA was detected intermittently and vanA remained below the detection limit in all samples. The total ARG marker abundances decreased by log 1.77 (p<0.05) in the plant using an aerated lagoon (AL), and by 2.69 logs (p<0.05) through treatment in the plant employing a biological nutrient removal (BNR) system. The BNR and secondary clarifier steps in both plants afforded the most removal of ARGs. The relative abundance of ARGs remained unchanged at the AL plant and showed a decreasing trend at the BNR plant. Levels of CBOD₅, nitrate and the human Bacteroides fecal marker correlated with ARG concentrations, suggesting these variables may be useful in predicting ARG removal. In conclusion, the effluent coming from the WWTPs contained eight of the studied ARG markers in concentrations ranging from 0.01 to 3.6 log copies/mL, indicating their release into the environment, however, the relative abundance of ARGs was not enriched during treatment in the two WWTPs.
1 Introduction

The overuse and misuse of antibiotics in medical and agricultural settings have resulted in their continuous release into the environment, which has contributed to the development of antibiotic resistant bacteria due to the selective pressure of the antibiotic residues (Davies and Davies, 2010). The efficacy of antibiotics may be reduced if antibiotic resistant genes (ARGs) are transferred to dangerous pathogens. Even in the absence of selective pressures, ARGs can persist in the environment and have been found in naturally occurring bacteria (Allen et al., 2010; Martinez, 2009; Tamminen et al., 2011). However, environments that are influenced by anthropogenic activities show higher concentrations of antibiotic resistant bacteria and ARGs (Allen et al., 2010; Pruden et al., 2006). Internationally, ARGs and antibiotic residues have now been recognized as emerging environmental pollutants (Pruden et al., 2006; Sanderson et al., 2016).

The effluent from wastewater treatment plants (WWTPs) is thought to be a major anthropogenic source for the release of antibiotic residues, antibiotic resistant bacteria, and ARGs into the environment (Czekalski et al., 2012; Munir et al., 2011; Rizzo et al., 2013). Although WWTPs are designed to remove carbonaceous materials, nutrients and pathogenic bacteria, they are not specifically designed to remove antibiotics and resistance elements (Barancheshme and Munir, 2018). Many ARGs are located on integrons, transposons, or plasmids, which allow mobilization and transfer to other bacteria (Allen et al., 2010). Biological wastewater treatment, due to the presence of large microbial communities, may create an ideal environment for selection and/or horizontal gene transfer of ARGs. Contributing factors include the close proximity of bacteria
originating from multiple sources, as well as a selective, nutrient-rich environment containing both antibiotics and heavy metals (Baquero et al., 2008; Davies and Davies, 2010; Levy and Marshall, 2004; Rizzo et al., 2013). Therefore, due to the risk of ARG enrichment during the treatment process and subsequent discharge into the environment, understanding wastewater related reservoirs is crucial to assessing the risk of antibiotic resistance transfer in the natural environment.

Antibiotics, antibiotic resistant bacteria and ARGs can be affected differently within WWTPs due to variations in the operations of the plant (Batt et al., 2007; Rizzo et al., 2013; Barancheshme and Munir, 2018). Many previous studies have primarily focused on influent and effluent samples, without analyzing the impact of individual steps in wastewater treatment processes (Laht et al., 2014; Narciso-da-rocha et al., 2014; Rodriguez-Mozaz et al., 2015). In order to better design plants to increase removal of resistance determinants and decrease their subsequent release into the environment, it is important to assess the effect of each step in the treatment process. This has therefore been the focus of more recent studies (e.g., Di Cesare et al., 2016, Lee et al., 2017). In Canada, WWTP effluent quality is monitored through measurement of parameters such as total suspended solids (TSS), carbonaceous biological oxygen demand (CBOD₅), or nutrient levels (Wastewater Systems Effluent Regulations, Environment Canada, 2015). Currently, neither antibiotics nor resistance elements are monitored for regulatory compliance purposes. Past studies have found significant correlations between the removal of nutrients and organic matter throughout treatment, and ARG concentrations (Nõlvak et al., 2013; Novo and Manaia, 2010). Therefore, examining these correlations may aid in finding potential proxies for monitoring ARG levels in treated wastewater.
The objective of this study was to examine how the individual treatment steps in two tertiary WWTPs, using different biological treatment operations, affected the abundance of nine ARGs, and one genetic indicator of mobility potential of ARG cassettes (intI1 integrase). A collection of water quality parameters (Escherichia coli, ammonia, phosphorus, CBOD5, HF183 human Bacteroides marker, bacterial 16S rRNA gene copy numbers, etc.) and presence of selected antibiotics were also assessed in order to examine their potential relationships to ARG abundances.

2 Materials and Methods

2.1 Treatment plants and sample collection sites

Samples were collected from two different types of tertiary WWTPs in Atlantic Canada; an aerated lagoon (AL plant) system, and a biological nutrient removal (BNR) system (BNR plant). The AL plant was sampled four times (July 7 and 28, and August 18, 2015, and February 23, 2016). The BNR plant was sampled on July 12, 2016 and July 18, 2017.

2.1.1. AL Plant

The AL plant discharges approximately $3.8 \times 10^6$ L/day of treated effluent into a river. The WWTP services around 600-800 customer connections including a Canadian Forces airbase, which contains a small hospital unit. There would also be several small commercial operations in the sewershed. Treatment steps include screening, two mechanically aerated lagoons (no recycling), two clarifiers, sand filtration, and ultra
violet light (UV) disinfection. The hydraulic retention time in the lagoons ranges between 18 and 24 hours (personal communications, plant operator), the organic loading rate to the lagoons is 0.12 kg CBOD₅/m²/d, and the mixed liquor suspended solids (MLSS) is approximately 300 mg/L. The surface overflow rate of the clarifiers is 14 m/d. The UV system is a Trojan 3000 PTP (Trojan Technologies, London, ON, Canada) with a design UV dose of 50 mJ/cm². The plant is designed to produce effluent with a maximum of 5 mg/L for CBOD₅ and 5 mg/L for TSS. Duplicate water samples were collected in pre-sterilized 1 L Nalgene collection bottles (Thermo Fisher Scientific, Waltham, MA, United States) after the screen (A) [referred to as the influent as it was the first accessible sampling site], after the mechanically aerated lagoons where water from the two lagoons was mixed (B), after the clarifiers where the effluent from two clarifiers was mixed (C), after sand filtration (D) and after UV disinfection (E) as shown in Figure 1A. Water samples were kept on ice while being transported back to the laboratory at Dalhousie University in Halifax, NS (a distance of approximately 150 km), stored at 4°C overnight and processed the following morning.

2.1.2 BNR Plant

The BNR plant services approximately 13,000 people in an urban area, including a local hospital, and light commercial and industrial operations. The effluent discharge of approximately $11.4 \times 10^6$ L/day is released into a harbour. The BNR plant uses the modified Johannesburg process, which is designed with activated sludge and sequential anaerobic, anoxic and aerobic reactor zones to remove ammonia, phosphorus, and nitrogen. Treatment steps include primary clarifiers, BNR process reactors, two
secondary clarifiers, and UV disinfection. The hydraulic retention time in the BNR process reactors is approximately 21 hours (personal communication, plant operator). The design MLSS and sludge age in the BNR process reactors are 3500 mg/L and 15 days, respectively. The surface overflow rates of the primary and secondary clarifiers are 30 and 15 m/d, respectively. The UV system is a Wedeco Model TAK55L (Xylem, Rye Brook, NY, USA) operated at a UV dose of 250 mJ/cm². The plant is designed to produce effluent with maximum concentrations of 10 mg/L for CBOD₅, 10 mg/L for TSS, 10 mg/L Total Nitrogen (TN) and 1 mg/L Total Phosphorus (TP). Duplicate samples were collected in pre-sterilized 1 L Nalgene collection bottles (Thermo Fisher Scientific) and collected from the raw influent (A), after the primary clarifiers (B), after BNR treatment (C), after the secondary clarifiers where out-going water from the two clarifiers was mixed (D), and after UV disinfection (E) as shown in Figure 1B. Samples were kept on ice and transported back to the laboratory at Dalhousie University in Halifax, NS (a distance of approximately 300 km) for processing the following morning.

2.2 Analysis of the Abundance of Antibiotic Resistance Genes, Human Fecal Markers and 16S rRNA Gene Copies

2.2.1 Genomic DNA extraction

Microorganisms in wastewater samples (30 to 500 mL) were concentrated either by filtration through 0.45 μm pore size membranes using a vacuum manifold (Millipore, Inc., Bedford, MA) or centrifugation at 3200 × g for 10 minutes. Filters or pellets were stored at -20°C until DNA extraction. Genomic DNA (gDNA) was extracted from the entire filter or cell pellet using the MoBio Powersoil DNA extraction kit (VWR
International, Ville Mont-Royal, QC, Canada) according to the manufacturer’s specifications. The concentration and purity of the DNA were evaluated by ultraviolet absorbance spectrophotometry at 260/280 nm and 260/230 nm (Implen Nanophotometer, Implen, München, Germany).

2.2.2. Quantitative real-time PCR

Primer and TaqMan hydrolysis probe sequences and cycling conditions for nine ARGs and one integrase gene were obtained from literature and are listed in Table S1 in the Supplemental Material. The genetic targets included class 1 integrase (Intl1), class A β-lactamase (blaCTX-M and blaTEM), erythromycin resistance gene (ermB), fluoroquinolone resistance gene (qnrS), sulphonamide resistance genes (sul1 and sul2), tetracycline resistance gene (tet(O)), methicillin resistance gene (mecA), and vancomycin resistance gene (vanA). These ARGs were chosen to represent a variety of different antibiotic classes and resistance mechanisms as well as clinically relevant genes (Szczepanowski et al., 2009; Volkmann et al., 2004).

The content of bacterial 16S rRNA genes (16S rRNA) was determined by qPCR (reaction conditions described in Table S1) to enumerate the total bacterial community and to allow for the calculation of the relative abundance of ARG relative to the 16S rRNA copy numbers. The human-specific fecal HF183 Bacteroides 16S rRNA genetic marker (HF183) (Sauer et al., 2011) was also quantified to examine a potential correlation between this DNA marker and ARG abundance. All targets were quantified in all water samples, except for one sampling run (AL plant, July 28, 2015) where tet(O), mecA, vanA and HF183 were not tested due to limitations in sample DNA quantities.
Control plasmids for \textit{intI1}, \textit{bla}\textsubscript{TEM}, \textit{sul1}, and \textit{sul2} were obtained from Dr. E. Topp (University of Western Ontario, London, ON, Canada) and described in Rahube et al. (2014a). Control plasmids for \textit{bla}\textsubscript{CTX-M}, \textit{ermB}, \textit{qnrS}, \textit{tet(O)}, \textit{mecA}, and \textit{vanA} were described in Neudorf et al. (2017). Concentrations of plasmid DNA were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). Standard curves were constructed for each assay using tenfold serial dilutions of plasmid controls and triplicate samples. Quality assurance for standard curves were performed using recommendations from Biorad Real-Time PCR Applications Guide (BioRad, 2013). Efficiencies ranged from 87% to 111% and $R^2$ values were >0.99 for all standard curves (Table S1). Limits of quantification (LOQ) (copies/reaction) were as follows: \textit{intI1}=14.4, \textit{sul1}=11.7, \textit{sul2}=9.6, \textit{tet(O)}=69.0, \textit{ermB}=13.8, \textit{bla}\textsubscript{CTX-M}=6.2, \textit{bla}\textsubscript{TEM}=243.0, \textit{qnrS}=112.0, \textit{mecA}=69.0, \textit{vanA}=138.0, 16S rRNA=67000, \textit{HF183}=3630. Limit of detection (LOD) was 5 copies/reaction (or 1 copy/mL for 500 mL sample volumes and 10 copies/mL for 50 mL sample volumes).

TaqMan qPCR assays for the ARG markers were performed on a Bio-Rad CFX96 Touch system (Bio-Rad, Hercules, CA, USA). The following reaction mixture was used: 1 x SsoAdvanced\textsuperscript{TM} Universal Probes Supermix (Bio-Rad), 0.9 μM of each primer, 0.25 μM TaqMan probe, 2 μL template DNA, and 2 μL of sterile nuclease-free water (Thermo Fisher) to a final volume of 20 μL. A Taqman qPCR assay was also used in quantification of the human faecal \textit{HF183 Bacteroides} marker, where the following reaction mixture was used: 1 x SsoAdvanced\textsuperscript{TM} Universal Probes Supermix (Bio-Rad), 0.6 μM of each primer, 0.25 μM TaqMan probe, 2 μL template DNA, and 6.5 μL of sterile nuclease-free water (Thermo Fisher Scientific) to a final volume of 25 μL. SYBR
Green qPCR was used to quantify bacterial 16S rRNA gene copy numbers and for this assay, the following reaction mixture was used: 1x Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, United States), 0.4 μM primers, and 1 μL of template DNA, and 7.4 μL of sterile nuclease-free water (Thermo Fisher Scientific) to a final volume of 20 μL. Samples and negative controls (no template DNA) were analyzed in duplicate, while standards (control plasmid) reactions were analyzed in triplicates.

Raw fluorescence data from the Bio-Rad CFX96 Touch system were imported into the LinRegPCR program (v. 11.4) (Ruijter et al., 2009). For each primer set and qPCR run, the fluorescence threshold was set to obtain a cycle threshold (CT) value for the positive control that matched the concentration specific CT value from the previously established standard curve. The LinRegPCR program automatically calculated the individual sample’s CT and $E_f$ (efficiency estimated from fluorescence increase) values.

In order to account for differences in efficiencies between samples and standards, a one-point calibration method for absolute quantification was used (Brankatschk et al., 2012).

For statistical analyses, any values that fell between the LOD and LOQ were set to $\frac{1}{2}$ LOQ value while values that fell below the LOD were set to $\frac{1}{2}$ LOD. For calculation of the relative abundance of genes, the gene copy number of each gene was normalized to the gene copy number of the 16S rRNA gene in each sample and log transformed (log(ARG gene copies/16S rRNA gene copies)). For calculation of the absolute abundance of genes, gene copies were normalized to the water volume used for gDNA extractions to generate gene copies per mL of water. For the AL plant, the calculation of absolute abundance was done for samples from August and February.
2.3 Assessment of Water Quality

Water samples were analysed within the recommended holding times of 24-48 hours for the following water quality parameters: CBOD₅, TSS, volatile suspended solids (VSS), TN, ammonia, nitrate, TP, chemical oxygen demand (COD), total coliforms, and *E. coli*.

Analysis of CBOD₅ was performed in duplicate according to the APHA standard method 5210B (American Public Health Association, 2005). TSS and VSS were performed using Whatman™ 934-AH 47 mm glass fiber filters (Thermo Fisher Scientific) according to APHA standard methods 2540 D (American Public Health Association, 2005). TN was analyzed using Hach® TN Test ‘N Tubes™ (0.5 to 25.0 mg/L N, Hach Company, Loveland, CO, United States), according to the manufacturer’s procedure. Ammonia and nitrate were measured using high performance ammonia and nitrate electrodes, respectively, as directed by the manufacturer (Thermo Fisher Scientific). The electrodes were attached to an Orion Star™ series meter (Thermo Fisher Scientific). TP was analyzed using Hach® TP Test ‘N Tube™ ranging from 1.0 to 100.0 mg/L PO₄³⁻ (Hach Company), following the manufacturer’s procedure. COD was analyzed using Hach® COD TNT plus Vial™ Test ranging from 20 - 1500 mg/L COD (Hach Company), following the manufacturer’s procedure. Total coliforms and *E. coli* were enumerated using IDEXX Colilert®-18 and Quanti-Trays® (IDEXX Laboratories, Inc., Westbrook, ME, United States) according to the manufacturer’s instructions (IDEXX Laboratories, Inc., 2012).
2.4 Detection of Antibiotics in Water Samples

Water samples from the August and February sampling events at the AL plant and from both sampling events at the BNR plant were tested for the content of 10 antibiotics (amoxicillin, cefaclor, cefprozil, cefdinir, levofloxacin, ciprofloxacin, azithromycin, clindamycin, clarithromycin, and triclocarban) at Acadia University (Wolfville, NS, Canada).

2.4.1 Sample Preparation for Antibiotic Detection

The pH of the samples (100 mL) was adjusted to pH 2.5±0.5 with 1 M HCl. The samples were then pumped (5 mL/min) through a vacuum filter apparatus equipped with 3 mL Chromabond® HR-X (200 mg sorbent) solid phase extraction columns, which had been pre-conditioned with 6 mL methanol and 6 mL of deionized (DI) water. Sample cartridges were then washed with 3 mL of 10% (v/v) methanol in DI and dried for 5 min under vacuum followed by the elution of analytes with 6 mL of methanol. Eluents were collected and reduced to 1 mL with gentle nitrogen blowdown at 37°C.

2.4.2 Quantification of Antibiotics

Antibiotic analysis was performed using an Agilent 1200 HPLC (Santa Clara, CA, USA) coupled with an Agilent 6410B triple quadrupole mass spectrometer. Chromatographic separation was performed using a 15-cm Agilent Poroshell Eclipse C18 column with a 4.6-mm internal diameter and 2.7-μm particles. The mobile phase consisted of 0.1% (v/v) formic acid buffer. The flow rate of mobile phase was 0.5 mL/min. A solvent gradient was programmed to start at 20% (v/v) methanol for 30
seconds, which increased to 100% (v/v) by 20 minutes. The column was held at a constant temperature of 40 °C. Following separation, ionization was conducted with an electrospray ionization source under 35-psi nebulizer pressure. Drying gas temperature was set to 350 °C with a flow rate of 12 L/min. The mass spectrometer was operated in the positive ion mode and the capillary voltage was held at 4000 V. Nebulizing gas and collision gas consisted of 98% nitrogen and ultra-high purity (UHP, 99.999 %) nitrogen, respectively. Precursor-to-product ion transitions were established for all target antibiotics. To boost the sensitivity of analysis, only one transition was monitored for each compound. Fragmentor voltage and collision energy were carefully optimized to achieve maximum response for each transition. The mass spectrometer parameters and transitions are shown in the supplementary information Table S2.

2.4.3 Quality assurance and quality control

To assist with quality assurance and quality control, solvent blank samples were included. Sampling bottles were pre-cleaned by manufacturers. These bottles and other glassware were thoroughly cleaned using DI water and methanol. Salinization of glassware was not performed because spiked samples did not show loss of target compounds.

Laboratory control samples were prepared by spiking a 100 mL DI sample containing 5 g of sodium chloride with an antibiotic mixture and subjecting the sample to the same analytical method. The recoveries of the target antibiotics were analyzed and reported. An internal standard was used to ensure the quality of instrumental analysis and
sample preparation. Duplicates were included to verify the reproducibility of the analytical method.

2.5 Data Analysis

One-way analysis of variance (ANOVA), Tukey HSD test, and t-tests were used to assess statistically significant differences (p<0.05) among samples. Log reductions were calculated as the log difference between treatment steps or influent/effluent. Pearson correlation coefficients were calculated (after performing check of normality) to assess if significant (p<0.05) correlations existed between the total ARG marker concentrations and water quality parameters. Analysis was performed using GraphPad Prism version 7.00 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com), and Microsoft Excel, 2016. The total ARG marker concentrations were calculated as the average of the sum of all tested ARG marker concentrations in the samples for each individual sample site and run.

3 Results and Discussion

3.1 The absolute abundance of ARGs during treatment in the AL and BNR plants

Measurements of water quality parameters and content of ARGs revealed no significant differences (p>0.05) between sample runs, indicating consistent performance of the WWTPs. For further analysis, the averages of the results from all sample runs are presented.
All ARG gene targets, with the exception of *mecA* (only intermittent detection) and *vanA* (never detected above LOD), were consistently detected in the municipal wastewater arriving at both WWTPs (Figures 2 and 3). Other WWTP studies have reported detection of *mecA* and *vanA*, which confer resistance to methicillin and vancomycin, respectively, both of which are antibiotics of last resort (Narciso-da-rocha et al., 2014; Szczepanowski et al., 2009; Volkmann et al., 2004). The low levels or absence of these targets in this study may be due to the fact that these WWTPs were servicing smaller urban centres (< 15,000 people). Furthermore, *mecA* and *vanA* genes can be present on chromosomal DNA as opposed to plasmids (Biavasco et al., 2007; Cetinkaya et al., 2000; Colomer-Lluch et al., 2011; Katayama et al., 2000) resulting in a limited range of host bacteria with lower presence/survival in the WWTP environment (Goldstein et al., 2012). ARGs belonging to the *tet* and *sul* families were detected in all influent samples and represent resistance genes to some of the oldest and most commonly consumed antibiotics (Al-Jassim et al., 2015; Auerbach et al., 2007; Du et al., 2015; Laht et al., 2014; LaPara et al., 2011). Other less studied ARGs (*qnrS, ermB, blaCTX-M, blaTEM*) have also been previously detected in wastewater samples, and represent genes that are often plasmid-borne and confer resistance to the latest generations of antibiotics (Marti et al., 2013; Narciso-da-Rocha et al., 2014; Rodriguez-Mozaz et al., 2014).

Overall, the AL treatment continuum led to a significant (p<0.05) decrease in the total ARG marker abundance of 1.77 log copies/mL from the influent sewage (total ARG of 6.29 log copies/mL) to the treated effluent (total ARG of 4.52 log copies/mL (Figure 2A). Total ARG concentrations decreased by the following log copies/mL after each treatment step: 0.16 (AL), 1.54 (secondary clarifier), 0.26 (sand filters), -0.20 (UV). This
showed that the secondary clarifiers were responsible for the largest incremental removal of ARGs, an observation agreeing with the result from the BNR plant (see below) and WWTP2 in the study of Lee et al. (2017). This removal may likely have occurred through the sedimentation processes and may imply that the generated biosolids contain a large amount of the ARGs, as has previously been observed (Börjesson et al., 2010; Chen and Zhang, 2013; Mao et al., 2015; Nnadozie et al., 2018). The AL, sand filters and UV treatment steps contributed little to the removal of ARGs in this WWTP.

In the AL plant, the content of individual genes exhibited, with the exception of \( sul_1 \) and \( bla_{CTX-M} \), significant (p<0.05) decreases from 0.94 log copies/mL (\( mecA \)) to 2.23 log copies/mL (\( qnrS \)), when comparing the influent and effluent water samples (Figure 2A). Previous research has suggested that sulfamethoxazole-resistant bacteria are difficult to remove (Gao et al., 2012), which may explain the insignificant reduction of \( sul_1 \). The significant (p<0.05) 1.74 log copies/mL decrease in the AL plant’s absolute abundance of 16S rRNA gene from 9.23 log copies/mL in the influent to 7.49 log copies/mL in the effluent (Figure 2C) was similar to the decrease in the total ARG concentration. Individual ARG concentration levels ranged from 3.27 (\( mecA \)) to 5.85 (\( qnrS \)) and from 2.32 (\( mecA \)) to 4.15 (\( int1 \)) log copies/mL in the influent and effluent samples, respectively.

In the BNR plant, there was a significant (p<0.05) decrease in total ARG concentration of 2.69 log copies/mL, from 7.01 in the raw influent to 4.33 log copies/mL in the effluent (Figure 3A). After the individual treatment steps, the total ARG concentration decreased by the following log copies/mL: 0.15 (primary clarifier), 1.36 (BNR), 0.62 (secondary clarifiers), 0.56 (UV). This means that in comparison to the AL
plant, the total ARG removal in the BNR plant was 0.92 log copies/mL or 88% higher (2.69 vs. 1.77 log copies/mL). Moreover, the BNR treatment step was responsible for the largest incremental removal of ARGs in the wastewater system; this was in contrast to the minimal removal observed in the biological treatment unit (i.e., the mechanically aerated lagoons) of the AL plant. The finding that the BNR plant’s sequential use of anaerobic, anoxic and aerobic reactors improved the removal of ARGs concurs with reports by Munir et al. (2011), Du et al. (2015) and Lee et al. (2017), who studied WWTPs with similar combinations of reactors with different oxygen conditions. Both the secondary clarification and the UV treatment in the BNR plant contributed significantly (p<0.05) to the removal of ARGs. The UV doses were 50 and 250 mJ/cm² in the AL and BNR plants, respectively, which likely explained the larger ARG log removal of 0.56 after this step in the BNR plant as compared to no decrease at the AL plant. In agreement with our observations, Lee et al. (2017) reported no decrease in ARGs following UV disinfection at a dosage of 27 mJ/cm² in two different WWTPs, while Zhang et al. (2015) reported similar log reductions for various ARGs after a UV dose of 250 mJ/cm².

Decreases in individual ARG genes from influent to effluent ranged from 2.20 (mecA) to 3.26 (blaCTX-M) log copies/mL. Interestingly, Helt (2012) and Börjesson et al. (2010) observed lower log removals of between 1.26 and 1.62 log copies/mL in a pilot and full scale BNR system, respectively, which were removal levels similar to those reported for the AL plant in the present study. The absolute abundance of the 16S rRNA gene decreased significantly (p<0.05) by 1.84 log copies/mL from 9.36 to 7.52 log copies/mL in the influent and effluent, respectively (Figure 2C). This indicated a lower relative reduction of the total bacterial population than of the ARGs, whose concentration
levels (log copies/mL) ranged from 2.21 (mecA) to 6.65 (intI1) in the influent and from 0.01 (mecA) to 4.09 (intI1) in the effluent.

3.2 Relative abundance of ARGs at the AL and BNR plants

At the AL plant, no significant differences (p>0.05) were observed in the relative abundance of the total ARG and the individual ARG markers between wastewater treatment steps, except for sul2, whose relative abundance significantly (p<0.05) increased after the lagoon step (Figure 2B). Hence, there was neither a relative enrichment nor reduction in the relative gene abundances through the AL treatment train, meaning that the total bacterial community (here represented by 16S rRNA gene copies) and antibiotic resistant bacteria were being reduced at comparable rates. As such it appeared that the AL plant neither selected for or against bacteria harbouring ARGs. This has, to the best of our knowledge, not previously been shown for AL type WWTPs.

Similar to the results from the AL plant, results from the BNR plant showed that the relative total ARG abundances remained unchanged (p>0.05) through the different treatment steps (Figure 3B). However, when examining each gene individually, the relative abundance of most genes tended to decrease after the BNR step (significant (p<0.05) for blaCTX-M), with the exception of sul2 and mecA, which increased slightly (Figure 3B) (also observed for sul2 at the AL plant). These results indicate that the BNR biological treatment may more efficiently decrease bacteria carrying ARGs than the AL treatment. It may be that the processes designed to remove nutrients may not favour bacteria harbouring the surveyed ARGs leading to the lower abundances of these bacterial communities after BNR treatment. The literature is still conflicting on whether
the relative abundance is changed in WWTPs as Mao et al. (2015) observed increases in  
the relative ARG abundance in two Chinese WWTPs while Di Cesare et al. (2016) and  
Bengtson-Palme et al. (2016) saw the opposite trend or no change in water samples from  
three Italian and three Swedish WWTPs, respectively. Besides varying operational  
parameters which make direct comparisons difficult, the nature of the raw wastewater  
may also be important. This was indicated in the study of two WWTPs using a series of  
anoxic, anaerobic and aerobic reactors as well as sedimentation, coagulation, filtration  
and UV, where the WWTP receiving predominantly municipal and industrial wastewater  
reported reduction in the relative abundance of several ARGs (\textit{tet, sul, erm, qnr, bla})  
while the relative abundance was enriched for \textit{sul, qnr} and \textit{bla} in the WWTP receiving a  
higher load of agricultural wastewater (Lee et al. 2017).

At both plants, \textit{intI1} became the most detected gene in the effluent (Figures 2A, B  
and 3A, B). This was also observed by Narciso-da-Rocha et al. (2014), who suggested  
that \textit{intI1} may be stable in wastewater. The detection of the gene marker for class I  
integrons in wastewater may provide a global perspective on antibiotic resistance and  
ARGs as integrons have the ability to carry and allow the transfer of multiple ARGs  
(Rizzo et al., 2013; Rahube et al., 2014b).

3.3 Changes in \textit{E. coli} and HF183 human \textit{Bacteroides} marker concentrations

Treatment at both plants significantly (p<0.05) decreased average \textit{E. coli} counts  
from 4.7-5.3 log MPN/mL in the influent to effluent values of <0.01 MPN/ mL (Figures  
2C and 3C). The human \textit{Bacteroides} fecal \textit{HF183} marker also decreased significantly  
(p<0.05) at both plants, with removals of 2.2 and 2.6 log copies/mL from the influent to
effluent water samples at the AL and BNR plants, respectively. Removal of *E. coli* and
the HF183 marker at the AL plant showed no correlation to the total ARG marker
concentrations (p>0.05), indicating no link between the removal of antibiotic resistance
related genes and *E. coli* or the HF183 marker. This was contrasted by the data from the
BNR plant, where the Pearson correlation coefficient (r=0.97) for the human HF183
marker in relation to total ARG marker concentrations was significant (p<0.05),
indicating that removal of the selected gene markers of antibiotic resistance and the
marker of human fecal contamination followed the same trend. Future research is
recommended to investigate the correlations between ARGs and *E. coli*/molecular fecal
markers in other WWTPs to assess the mechanisms and relevance of these indicators for
prediction of ARG removal.

Treatment in both the AL and BNR plants caused a 6 log MPN/mL decrease in
viable *E. coli* counts but only around 2-3 log decreases for ARGs, 16S rRNA and HF183
gene copies/mL. The use of qPCR for detection of genetic elements, such as ARGs and
HF183, to monitor removal or inactivation of fecal indicator bacteria in wastewater may
be problematic as the decrease in genetic markers may not follow decreases in viable
bacteria levels due to possible detection of markers from dead cells or cell-free DNA
(Chern et al., 2014; Zhang et al., 2018). This phenomenon is likely observed in Figures 2
and 3, where the UV disinfection step significantly reduced culturable *E. coli* counts,
while ARG, 16S rRNA and HF183 copies/mL remained unchanged. However,
exogenous DNA can still be transferred to other bacteria through transformation (Frost et
al., 2005), especially while in presence of selective pressures (e.g., antibiotics and
metals), therefore extracellular ARGs entering the environment through effluent
discharge may still present a possible threat to public health.

3.4 Water quality assessment and correlation to gene abundance

The AL plant was efficient at nutrient and solids removal as all measured
parameters decreased between 0.5 and 2 orders of magnitude (Table 1), with the
exception of nitrate which was increased due to conversion of ammonia into nitrate
(nitrification). The plant met the regulatory targets of 5 mg/L for both TSS and CBOD₅ in
the effluent. In regard to correlations between total ARG concentrations and water
quality at the tested sites, nitrate (r=-0.92, p<0.05) and CBOD₅ (r=0.95, p<0.05) showed
significant correlations (Table 1), suggesting that decreasing CBOD₅ and increasing
nitrate levels may help predict a decrease in ARGs in wastewater effluent from this type
of plant design.

The BNR plant also met its regulatory targets of reducing TSS and CBOD₅ to
below 25 mg/L with its efficient removal of nutrients and solids (Table 2). While there
was a trend toward COD, CBOD₅ and ammonia correlating with ARG removal, these
relationships were not statistically significant (p>0.05).

Relationships between removal of nutrients and ARG abundance have been
suggested in other studies (Börjesson et al., 2010; Du et al., 2015; Nõlvak et al., 2013;
Novo and Manaia, 2010). Similar to the AL plant, Nõlvak et al. (2013) found significant
correlations between nitrate (negative) and organic matter (positive) with ARG
concentrations. Further testing would be needed for the BNR plant to determine if the
trends observed would become significant.
3.5 Antibiotic Results and Correlations

All tested antibiotics, besides cefprozil and cefaclor, were detected in various samples from both plants (Supplemental material Tables S3 and S4). Of the tested penicillin antibiotics, only amoxicillin and cefdinir were detected despite their high consumption, which may be due to their chemical instability (Graham et al., 2011). The tested macrolides (azithromycin, clindamycin and clarithromycin) were detected in samples obtained after all treatment steps indicating their poor removal in both WWTPs as was also observed by Marx et al. (2015) and Bengtson-Palme et al. (2016).

Interestingly, both influent and effluent water samples from the AL plant showed higher concentrations of antibiotics compared to the BNR plant, a fact which warrants further investigations and may have contributed to the lower ARG removals in the AL plant.

Correlation analysis was performed and showed no significant (p<0.05) relationships between the individual ARG absolute concentrations and the antibiotics they confer resistance to (see Table S2). Other studies have found correlations in the past (Gao et al., 2012; Gao et al., 2015; Rodriguez-Mozaz et al., 2015), while Bengtson-Palme et al. (2016) found none. Possible correlations may depend on the class of antibiotics since their transport and/or susceptibility to degradation vary markedly (Marx et al., 2015; Nnadozie et al., 2017).
4 Conclusions

The tertiary WWTPs reduced effluent ARG marker concentrations by approximately 2 log copy/mL but were still releasing total ARG markers in the order of $1.3 \times 10^{14}$ and $2.8 \times 10^{14}$ copies/day for the AL and BNR plant, respectively. The best removal was obtained in the BNR reactors (modified Johannesburg process), which uses a series of reactors with anoxic, anaerobic and aerobic reactors, as opposed to the aerobic AL lagoons. The secondary clarifiers effected significant removal of ARGs in both WWTPs and did a high UV dose of 250 mJ/cm$^2$ in the BNR plant. The relative abundance of ARGs generally remained unchanged in samples taken along the entire treatment train of the AL and BNR plant, indicating that these types of wastewater treatment systems do not promote the enrichment of ARGs in the bacterial population. Changes in organic matter and the removal of the human fecal HF183 marker may be useful in predicting ARG removal, however, further testing is needed to solidify these relationships.

5 Acknowledgments

We would like to thank the local authorities for allowing us access to the two wastewater treatment plants. We would also like to thank our team members for their assistance in the field and laboratory (Audrey Hiscock, Robert Johnson, Amy Jackson, and Joanna Poltarowicz). Funding for this project was provided by the Natural Science and Engineering Research Council of Canada (STGP 463352 – 14).


Figure Legends.

Figure 1. Schematic layout of the treatment trains of A) the aerated lagoon (AL) plant and B) the biological nutrient removal (BNR) plant with sampling sites indicated by letters (A-E).

Figure 2. Effect of individual treatment steps in an mechanically aerated lagoon wastewater treatment plant on the absolute and relative abundances of ARGs and fecal indicators in water samples (averages ± standard deviations). a) Absolute abundances of each ARG, intI1 and the sum of all ARG markers (Total ARG) (n=4). b) Relative abundances of each ARG, intI1 and Total ARG (n=8). c) Concentration of 16S rRNA genes, E. coli and the human HF183 Bacteroides marker (n=4). (Sand is an abbreviation for the sand filter)
Figure 3. Effect of individual treatment steps in a biological nutrient removal reactor wastewater treatment plant on the absolute and relative abundances of ARGs and fecal indicators in water samples (n=4, averages ± standard deviation). a) Absolute abundances of each ARG, \textit{intI1} and the sum of all ARG markers (Total ARG). b) Relative abundances of each ARG, \textit{intI1} and Total ARG. c) Concentration of 16S rRNA gene copies, \textit{E. coli} and the human HF183 \textit{Bacteroides} marker. (1\textsuperscript{st} and 2\textsuperscript{nd} refer to the primary and secondary clarifiers).
## Tables.

Table 1. AL plant water quality data and Pearson correlation coefficients (average results ± standard deviations from four sampling events).

<table>
<thead>
<tr>
<th></th>
<th>Nitrate (mg/L)</th>
<th>Ammonia (mg/L)</th>
<th>TN (mg/L)</th>
<th>TSS (mg/L)</th>
<th>VSS (mg/L)</th>
<th>COD (mg/L)</th>
<th>TP (mg/L)</th>
<th>CBOD\textsubscript{5} (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent</td>
<td>1.5</td>
<td>18.8</td>
<td>31.3</td>
<td>160.9</td>
<td>151.2</td>
<td>406.1</td>
<td>9.4</td>
<td>169.3</td>
</tr>
<tr>
<td>±1.7</td>
<td>± 5.8</td>
<td>± 6.76</td>
<td>± 63.3</td>
<td>± 59.6</td>
<td>± 70.3</td>
<td>± 2.1</td>
<td>± 42.2</td>
<td></td>
</tr>
<tr>
<td>Lagoon</td>
<td>5.5</td>
<td>1.0</td>
<td>201.1</td>
<td>2861.3</td>
<td>2332.0</td>
<td>4272.3</td>
<td>255.6</td>
<td>242.3</td>
</tr>
<tr>
<td>± 2.7</td>
<td>± 0.55</td>
<td>± 41.83</td>
<td>± 1006.0</td>
<td>± 812.5</td>
<td>± 1478.0</td>
<td>± 69.0</td>
<td>± 92.3</td>
<td></td>
</tr>
<tr>
<td>Clarifier</td>
<td>8.1</td>
<td>0.7</td>
<td>10.5</td>
<td>3.9</td>
<td>4.8</td>
<td>21.9</td>
<td>3.3</td>
<td>3.6</td>
</tr>
<tr>
<td>± 3.7</td>
<td>± 0.61</td>
<td>± 7.57</td>
<td>± 1.9</td>
<td>± 1.2</td>
<td>± 5.4</td>
<td>± 3.7</td>
<td>± 3.3</td>
<td></td>
</tr>
<tr>
<td>Sand Filter</td>
<td>8.6</td>
<td>0.3</td>
<td>7.5</td>
<td>1.6</td>
<td>1.4</td>
<td>21.3</td>
<td>3.1</td>
<td>2.9</td>
</tr>
<tr>
<td>± 3.6</td>
<td>± 0.40</td>
<td>± 5.00</td>
<td>± 1.2</td>
<td>± 0.66</td>
<td>± 0.50</td>
<td>± 3.7</td>
<td>± 1.3</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>8.3</td>
<td>0.1</td>
<td>6.7</td>
<td>1.6</td>
<td>1.7</td>
<td>18.9</td>
<td>3.2</td>
<td>1.3</td>
</tr>
<tr>
<td>± 3.4</td>
<td>± 0.39</td>
<td>± 4.00</td>
<td>± 0.66</td>
<td>± 0.52</td>
<td>± 2.0</td>
<td>± 3.8</td>
<td>± 1.3</td>
<td></td>
</tr>
<tr>
<td>Pearson correlation coefficient (p-value) with ARG concentrations</td>
<td>-0.92 (p&lt;0.05)</td>
<td>0.69 (p&gt;0.05)</td>
<td>0.65 (p&gt;0.05)</td>
<td>0.60 (p&gt;0.05)</td>
<td>0.60 (p&gt;0.05)</td>
<td>0.62 (p&gt;0.05)</td>
<td>0.57 (p&gt;0.05)</td>
<td>0.95 (p&gt;0.05)</td>
</tr>
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</table>
Table 2. BNR plant water quality data and Pearson correlation coefficients (average results ± standard deviations from two sampling events).

<table>
<thead>
<tr>
<th></th>
<th>Ammonia (mg/L)</th>
<th>TN (mg/L)</th>
<th>TSS (mg/L)</th>
<th>VSS (mg/L)</th>
<th>COD (mg/L)</th>
<th>TP (mg/L)</th>
<th>CBOD₅ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw influent</td>
<td>26.2 ± 6.22</td>
<td>39.4 ± 13.3</td>
<td>309.5 ± 16.3</td>
<td>215.15 ± 5.44</td>
<td>607.0 ± 504.87</td>
<td>31.6 ± 25.7</td>
<td>302.3 ± 215.00</td>
</tr>
<tr>
<td>Primary Clarifier</td>
<td>14.7 ± 2.40</td>
<td>24.7 ± 2.92</td>
<td>70.8 ± 35.6</td>
<td>55.5 ± 29.00</td>
<td>327.3 ± 228.16</td>
<td>15.4 ± 2.07</td>
<td>225.8 ± 2.37</td>
</tr>
<tr>
<td>BNR</td>
<td>0.66 ± 0.39</td>
<td>250.5 ± 126.6</td>
<td>3201.5 ± 1839.2</td>
<td>1933.8 ± 690.5</td>
<td>1369.5 ± 976.5</td>
<td>&gt;186.0 ± 81.3</td>
<td>462.3 ± 44.9</td>
</tr>
<tr>
<td>Secondary Clarifier</td>
<td>0.11 ± 0.10</td>
<td>3.6 ± 0.57</td>
<td>3.3 ± 1.27</td>
<td>4.3 ± 2.97</td>
<td>40.0 ± 11.3</td>
<td>0.10 ± 0.07</td>
<td>6.5 ± 4.16</td>
</tr>
<tr>
<td>UV</td>
<td>0.14 ± 0.15</td>
<td>4.0 ± 0.57</td>
<td>3.4 ± 1.70</td>
<td>1.6 ± 0.85</td>
<td>18.0 ± 2.83</td>
<td>0.00 ± 0.00</td>
<td>5.6 ± 3.45</td>
</tr>
<tr>
<td>Pearson correlation coefficient (p-value) with ARG concentrations</td>
<td>0.83 (p&gt;0.05)</td>
<td>0.002 (p&gt;0.05)</td>
<td>0.005 (p&gt;0.05)</td>
<td>-0.0001 (p&gt;0.05)</td>
<td>0.32 (p&gt;0.05)</td>
<td>0.0035 (p&gt;0.05)</td>
<td>0.34 (p&gt;0.05)</td>
</tr>
</tbody>
</table>
Figure 1. Schematic layout of the treatment trains of A) the aerated lagoon (AL) plant and B) the biological nutrient removal (BNR) plant with sampling sites indicated by letters (A-E).
Figure 2

Click here to download high resolution image
Figure 3
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